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Cell-Based Memory of DNA Damage in Breast Cancer

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14. ABSTRACT <p>Although there is a large range of cancer phenotypes, observed even among breast cancers, it has often been proposed that most or all cancers share a basic mechanism of progression to malignancy. A popular carcinogenesis model framework divides the progression into three stages: initiation, promotion and progression. In the earliest stages, comprising initiation, cells are thought to shift into a physiological state that is predisposed to developing the mutations and characteristics necessary for malignancy. The events of initiation – whether they are similar for all cancers or not – likely hold the key to truly understanding how and why normal cells become cancerous. It is at the step of initiation that many anticarcinogenic compounds are thought to act. However, the process of initiation has been hard to study because of our inability to identify cells that can eventually become cancerous very early in the process and track them over time.</p> <p>The long-term goal of the research program is to define in detail the genetic and epigenetic changes in initiated cells that, over time, confer a predisposition to malignancy. We proposed to take a novel approach to this problem by building and characterizing a synthetic transcription-based memory circuit that will allow us to mark and track the lineages of single cells that have suffered transient DNA damage.</p> <p>For this proposal, we had two goals: 1) to construct a tunable mammalian cell-based memory device; and 2) to establish a genetic circuit to permanently record the experience of a transient DNA damaging event in a damaged cell and its progeny, and to use the output to collect specific cells for long-term tracking of phenotypic change. We made substantial progress on both Aims. We successfully constructed a model cell-based memory device mammalian cancer cells. We characterized a DNA damage sensitive promoter as well as several designer transcription factors. We have incorporated these factors into the memory device and established new ways to stably introduce them into tumor cells.</p>				
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## Introduction:

Cancer is a complex disease of unregulated growth. Although there is a large range of cancer phenotypes, observed even among breast cancers, it has often been proposed that most or all cancers share a basic mechanism of progression to malignancy. A popular carcinogenesis model framework divides the progression into three stages: initiation, promotion and progression. In the earliest stages, comprising initiation, cells are thought to shift into a physiological state that is predisposed to developing the mutations and characteristics necessary for malignancy. The events of initiation — whether they are similar for all cancers or not — likely hold the key to truly understanding how and why normal cells become cancerous. It is at the step of initiation that many anticarcinogenic compounds are thought to act. However, the process of initiation has been hard to study because of our inability to identify cells that can eventually become cancerous very early in the process and track them over time. The long-term goal is to define in detail the genetic and epigenetic changes in initiated cells that, over time, confer a predisposition to malignancy. A novel approach to this problem is to build and characterize a synthetic transcription-based memory circuit that will allow tracking of the lineages of single cells that have suffered transient DNA damage. We expect the cells that mount a repair-oriented survival response are the most likely initiated cellular sub-population. Enriching specific populations of cells from a heterogeneous pool will enable more detailed and accurate analysis of the initiated cell state, such as through gene expression and epigenetic profiling.

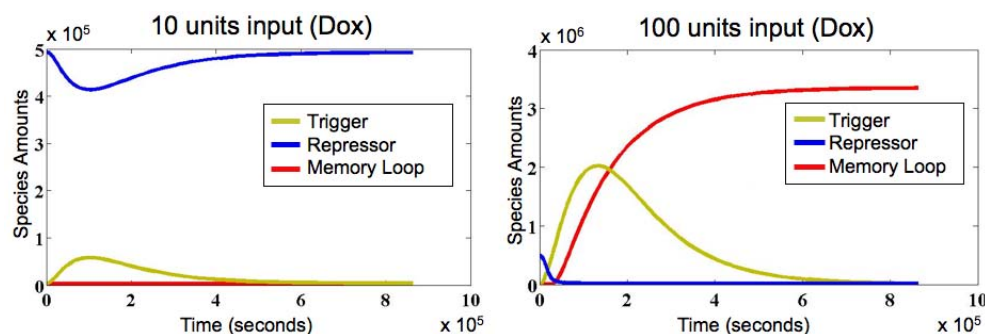
## Body:

### Aim 1.

Task 1: Creation of an inducible memory device in stable cell lines

A synthetic positive feedback memory circuit has been established in mammalian cells. This circuit echoes the work performed in yeast [Ajo-Franklin et al., 2007]. A set of transcriptional activators was constructed and stably transformed into U2OS cells. In the resting state, a constitutively expressed Repressor containing a KRAB domain inhibits spontaneous activation of the Memory Loop gene by binding upstream. Upon addition of a threshold amount of Doxycycline, the Trigger transcriptional activator competes for binding sites upstream of the Memory Loop gene. The Trigger transcript encodes a miRNA to repress expression of the Repressor. The activated Memory Loop also produces miRNA against the Repressor; this leads to cooperative inhibition of the Repressor and stable positive feedback of the Memory Loop.

A mathematical model for the memory circuit was developed based on published general rate constants and equations for the processes involved (transcription activation, splicing, translation, degradation, etc.). Model output suggests that the constitutively expressed Repressor confers a high activation threshold for the circuit.



**Modeling of the mammalian memory circuit (Dave Drubin).** Computer modeling output. The input threshold for activation is such that the Memory Loop is not sensitive to low levels of stimulus. An increase in Trigger and Memory Loop gene expression cooperatively deactivates the Repressor.

Following the addition of Doxycycline to clonal transgenic cells, expression of the three fluorescently tagged proteins in the synthetic circuit was detected *in vivo* by fluorescence microscopy and flow cytometry. Low levels of YFP indicate leaky expression of the Trigger in the uninduced state. Doxycycline increases expression of the Trigger protein, a reduction of the CFP-tagged repressor protein, and a modest increase in the RFP-tagged Memory Loop protein.

To test the circuit for positive feedback behavior, the cells were treated with a 24-hour pulse of the stimulus (doxycycline), washed, and imaged over two days in doxycycline-free medium. Leaky expression of the Trigger in the unstimulated state was eliminated using tetracycline-free serum. The Trigger showed transient expression that coincided with the addition and removal of doxycycline.

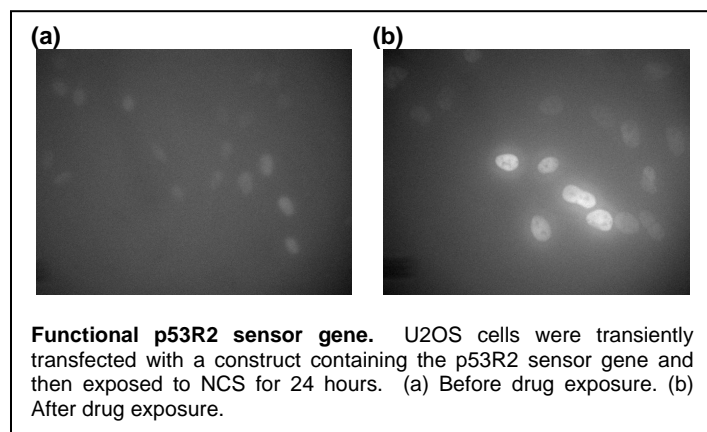
## Aim 2.

Task 1: Create a stable p53 response memory cell line.

To study the events of initiation and subsequent tumor progression, we are analyzing the cellular response to transient DNA damage. The tumor suppressor p53 plays a critical role in damage response by initiating a heterogeneous response in a cell-, tissue- and stress-dependent manner via transcriptional activation of cascades that promote genomic stability, DNA repair, cell cycle arrest, senescence, and apoptosis. One of the key p53 target genes is p53R2, which encodes a small subunit of ribonucleotide reductase (RNR), a complex that supplies nucleotides to repair damaged DNA. Expression of p53R2 is activated in a p53-dependent manner by  $\gamma$ - and UV-irradiation, as well as numerous genotoxic agents. Thus, linking p53R2 to the memory device will allow us to record a repair response to transient DNA damage that is associated with long-term phenotypic changes.

The proposed device will have the novel capability of specifically identifying and tracking cells that mount a repair response to DNA damage. These cells will be identified within a population that responds heterogeneously to damage: damage responses may include repair, genomic stability, arrest, senescence, or apoptosis. As a result, we may be able to capture a potentially initiated state and its progression in cell progeny. Such a tool will allow for the enrichment of a specific cell population for analysis, thereby preventing undesired cell populations from diluting out relevant results.

To this end, a DNA damage-responsive mammalian memory device is in the process of being engineered. P53R2 operators sequences followed by a commonly used TATA box from the HSV thymidine kinase gene, an intron sequence (to enhance gene expression), and a kozak sequence have been cloned to create the promoter for the trigger gene. Mammalian codon-optimized versions of the bacterial transcription factor LexA have been generated, and their expression and functionality have been demonstrated in U2OS cells. Similarly, mammalian codon-optimized versions of all six zinc fingers have been cloned and will subsequently be tested as transcription factors produced by the damage-inducible trigger gene. The p53R2 trigger gene has been verified by sequencing and transfected into U2OS cells using the Flp-In T-Rex Core Kit (Invitrogen), chosen because they have an active p53 response and high



transfection efficiency. In a preliminary experiment, a selected population of transfected cells was transiently exposed for 24 hours to neocarzinostatin (NCS), a p53-inducing drug that causes double-strand breaks in DNA. Fluorescence microscopy was then used to observe the response of these cells as compared to unexposed cells. As expected, a population of exposed cells expressed sensor protein in response to damaging agent.

We are actively pursuing the attachment of the p53R2 damage-inducible triggers to the memory loop, testing a variety of transcription factors for functionality and the use of insulator sequences to prevent heterochromatin formation (i.e. loss of gene function). As previously mentioned, we hypothesize that integrating the trigger and memory genes in the same genomic location, on the same plasmid, will be most efficient. Stable clones containing the complete memory circuit will ultimately be selected for functionality by FACS and fluorescence microscopy.

A preliminary mathematical model for the simple memory device and its tuning component has been developed, based on published general rate constants and equations for the processes involved. According to this model, there are definite regimes in which the memory device should be functional. The model also indicates that it will be beneficial to employ some techniques aimed at tuning or enhancing the bistability of the circuit. Specifically, it is likely that installation of the simple memory circuit in mammalian cells will produce some stable clones that exhibit expression in the absence of input signal. For example, leaky expression from either the sensor or autofeedback genes may allow for an accumulation of enough memory protein to trigger the self-sustaining feedback loop. The leakiness will depend upon the genes themselves as well as the chromatin context within which the gene integrates.

We are also taking a more careful approach to the integration of synthetic genes in the mammalian genome. One parameter we believe is most critical is the prevention of heterochromatin formation in the location of integration. To prevent this, we are now using a U2OS cell line containing a Flp-In system, such that we can target our genes of interest to a locus that has been previously determined to remain euchromatic. Furthermore, we are using "insulator sequences" (based on the insulator sequences found in the chicken B-globin gene) before, between, and after any integrated genes. Lastly, we have decided to place both the trigger and memory loop on the same plasmid, at least initially. This will enable us to target both genes to the same locus via the Flp-In system, so we know that gene expression will not be affected by differences in gene dosage; in the future, of course, we may want to play with differences in gene dosage. We are building versions of the device that do and do not contain

insulator sequences between the two tandem genes, to determine whether this has an effect on device function.

Another concern related to induction of the memory loop is that the strength and action of the transcription factor produced by the trigger may have a significant impact on the device's function as a whole. To address this issue, we are now testing not only full-length LexA, but also six different synthetic zinc fingers that have been shown to function as activators.

All mentioned versions of the trigger have been tested and shown to be functional. We are now at the point of connecting the triggers to their corresponding versions of the memory loop and integrating these into Flp-In cells.

## Aim 2.

### Task 2. Create a miRNA tuning device

Currently a new device has been built in mammalian U2OS cells to allow visualization of inducible miRNA expression. This device consists of a Doxycycline-inducible promoter attached to an intronic miRNA targeting luciferase, with mVenus added with a nuclear localization sequence to visualize promoter activation. When Doxycycline is added, nuclear localized mVenus expression directly correlates with miRNA expression, which silences luciferase reporter expression. Different miRNA can be inserted using a BbsI site into the intronic miRNA, allowing for generalized indirect correlation of miRNA expression.

### Key Research Accomplishments:

- Construction and modeling of a memory loop in cancer cells
- Characterization of a p53 DNA-damage sensitive promoter
- Introduction of the damage sensitive promoter into the memory device
- Engineering of designer transcription factors
- Creation of an miRNA tuning device for the memory circuits

### Reportable outcomes:

#### Publications

Burrill DR and Silver PA. Making cellular memories. *Cell* 2010 140:13-18.

#### Presentations

Burrill SR and Silver PA. NSF SYNBERC Annual Meeting 2010

#### Funding applied for:

NIH TRO1

### Conclusion

The major results from this one year grant include the engineering of a cell-based memory device that can be placed into cancer cells to track the initiation of DNA damage-associated events. Towards that end, we have characterized a DNA damage sensitive promoter and constructed several new designer transcription factors. In addition, we have incorporated a unique tuning device using miRNAs. Taken together, these results lay at the foundation of the application of Synthetic Biology to cancer studies and will be of widespread interest and utility.

From a cancer perspective, once completed, we will be able to track the fate of individual cells in animal tumor models and the affects of drugs on individual cells within tumors. This will have major impact in understanding the nature of therapeutic action within breast tumors.

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Ajo-Franklin CM, Drubin DA, Easking JA, Gee EP, Landgraf D, Phillips I and Silver PA. Rational design of memory in eukaryotic cells. *Genes Dev.* 2007. Sep 15:21(18):2271-6 PMID 17875664

Burrill DR and Silver PA. Making cellular memories. *Cell* 2010 140:13-18. PMID20085698